

Daidzein and Genistein produced by a marine *Micromonospora carbonacea* FIM 02-635

JIANG Hong^{1,2}, CHENG Yuan-rong^{1*}, ZHENG Wei¹

(1. Fujian Institute of Microbiology, Fuzhou 350007, China;

2. Fujian Medical University, Fuzhou 350004, China)

Abstract : Objective To study the active secondary metabolites from marine microorganism FIM02-635. **Methods** The producing strain was identified by taxonomical and phylogenetic studies. Two compounds FW635I1 and FW635I2 with immunosuppressive activities were extracted by organic solvents from the culture broth and purified by silica gel column chromatography and high speed counter current chromatography. The structures of the two compounds were determined by physico-chemical properties and spectral analyses, the biological activities were assayed *in vitro*. **Results and Conclusion** The producing strain was named as *Micromonospora carbonacea* FIM 02-635. Two compounds FW635I1 and FW635I2 were determined to be isoflavone Daidzein and Genistein, respectively, showed immunosuppressive and antitumor activities, but not antimicrobial activities.

Key words : *Micromonospora*; immunosuppressive; isoflavones

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海洋碳样小单孢菌产生的大豆黄素和染料木素

江红^{1,2}, 程元荣^{1*}, 郑卫¹

(1. 福建省微生物研究所, 福建 福州 350007; 2. 福建医科大学, 福建 福州 350004)

摘要 : 目的 研究海洋微生物 FIM02-635 产生的活性次级代谢产物。方法 对产生菌进行分类鉴定。发酵液用有机溶媒提取具有免疫抑制活性的化合物, 采用硅胶柱层析及高速逆流色谱的分离方法, 提取纯化到 2 个化合物 FW635I1 和 FW635I2, 通过理化性质测定和光谱学测定分析 2 个化合物的结构并对这两个化合物进行体外生物活性测定。结果与结论 产生菌定名为碳样小单孢菌 FIM02-635。FW635I1 和 FW635I2 分别与异黄酮大豆黄素和染料木素同质, 它们具有免疫抑制和抗肿瘤活性, 但没有抗菌活性。

关键词 : 小单孢菌属; 免疫抑制; 异黄酮

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In the course of screening for novel immunosuppressants from marine *Micromonospora*, two important isoflavones were isolated from the culture broth of a marine *Micromonospora* strain FIM 02-635.

This paper reports on the taxonomy and fermentation of the strain FIM 02-635, the isolation, physico-chemical properties

and the biological properties of two compounds.

1 Materials and Methods

1.1 Taxonomy Studies

A marine *Micromonospora* strain FIM 02-635 was isolated from a beach soil collected in Fujian Province, China. Morpho-

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* Corresponding author : E-mail fim@pub3.fz.fj.cn

logical cultural characteristics and physiological properties of strain FIM 02-635 were investigated by the methods of Shirling *et al* and Waskman^[1,2]. Cultures were observed after incubation at 35 °C for 2 weeks. Carbohydrate utilization was tested by grown on Pridham and Gottlieb's medium^[3] containing 2 % carbon source at 35 °C for 2 weeks.

The amino acid composition of the cell wall and the sugar composition of the whole cell were analysed by the method of Hasegawa^[4]. Phospholipids, menaquinones and mycolic acids were analysed by the method of Minnikin^[5] and Genomic DNA was determined by the method of De Ley^[6]. Phylogenetic study of the strain FIM 02-635 was made by analysis of the 16S rDNA gene base sequence.

1.2 Fermentation

A loop of culture was inoculated into a 500 mL flask containing 80mL of seed culture medium consisting of (%) soluble starch 1.5, glucose 0.5, peptone 0.5, yeast extract 0.5, (NH₄)₂SO₄ 0.05, K₂HPO₄ · 3H₂O 0.05, NaCl 0.05, MgSO₄ · 7H₂O 0.05, CaCO₃ 0.1, pH 7.5. Incubation was carried out at 32 °C for 48h on a rotary shaker operating at 240r · min⁻¹. After 2 days incubation, a 10 % of seed culture was transferred into a 500mL flask containing 80mL of fermentation medium consisting of (%) soluble starch 4, glucose 0.5, soybean meal 2.5, yeast extract 0.5, MgSO₄ · 7H₂O 0.05, K₂HPO₄ · 3H₂O 0.05, CaCO₃ 0.1, pH 7.5. Fermentation was performed at 32 °C for 4 to 5 days on a rotary shaker operating at 240 r · min⁻¹.

1.3 Isolation and purification

The harvested culture broth was centrifuged and the mycelia cake was extracted with ethanol twice. The ethanol extract evaporated under reduced pressure and the resulting solution was extracted with ethyl

acetate twice, concentrated in vacuum to a syrup. The syrup was chromatographed on a silica gel G column (4 × 50cm) and eluted with petroleum ether : acetone as a gradient eluting solvent. The same active portions analysed by HPLC were pooled and evaporated under reduced pressure. The resulting light yellow powder was dissolved in a small amount of methanol and purified by TBE-300 HSCCC (high speed counter current chromatography) using chloroform : methanol : water (4 : 3 : 2) as the solvent (lower phase as the mobile phase). The apparatus was rotated at 800 r · min⁻¹. Two active fractions, FW635I1 and FW635I2, were obtained and dried under reduced pressure respectively.

HPLC was performed on a column of ODS-C18 at 50 °C with an eluent of MeOH : H₂O 40 : 60 (v/v). The flow rate was 1mL per minute, the absorption of the effluent was monitored at 260nm. The retention time for FW635I1 was 10.2min, for FW635I2 was 10.7min, respectively.

1.4 Biological activities in vitro

1.4.1 Inhibition effect on splenocyte proliferation

Immunosuppressive activity was determined by lymphocyte growth inhibition. Splenocytes were prepared from Kunming mice and ConA induced splenocyte proliferation was performed in 96-well microtiter plates with each well containing 4 × 10⁶ splenocytes in 0.1mL RPMI1640 medium supplemented with 10 % fetal bovine serum, to which ConA (10μg · mL⁻¹) was added. The cells were incubated at 37 °C in a 95 % air : 5 % CO₂ incubator for 3 days. Growth was measured by the colorimetric MTT assay described by Mosmann^[7].

1.4.2 Inhibition of IL-2 Production

IL-2 in the ConA induced splenocyte supernatant was quantified with ELISA IL-2 Kit

purchased from Wuhan Bosder Company.

1.4.3 Antitumor activity *in vitro*

The human leukemia cell line K562 was grown in 96-well microplates in RPMI 1640 medium supplemented with 10% fetal bovine serum, cells were incubated at 37 °C in a 95% air 5% CO₂ incubator. The test samples in DMSO were diluted with the medium and added to the wells respectively. After incubation for 72h, the cytotoxicity against the tumor cell was determined by the colorimetric MTT assay.

1.4.4 Antimicrobial activity

Antimicrobial activities were determined by using 6mm paper discs and nutrient agar medium for bacteria, incubation at 37 °C overnight; and Sabroud's agar medium for yeast and fungi at 30 °C for 2 or 3days.

2 Results

2.1 Taxonomy of strain FIM 02-635

The vegetative mycelium of strain FIM 02-635 was 0.3 μm in diameter, developed, branched and septate. Spores were born singly on short sporophores, spherical or ovoid (0.6-0.7 μm × 0.6-0.9 μm) and the surface of spore was slightly rough (Fig. 1). Aerial mycelium was not observed. The vegetative mycelium was brownish or orange-brown and the soluble pigment was brownish or yellowish brown.

On the basis of morphology, culture characteristics, physiological properties and the chemotaxonomic characteristics (data not shown), strain FIM 02-635 was inferred to be a member of the genus *Micromonospora*. The complete 16S rDNA gene base (Accession No. GenBankA Y697299) sequence of strain FIM 02-635 was showed the strain highly associated with *Micromonospora carbonecea* (98.6% similarity). The strain was identified as *Micromonospora carbonecea* FIM 02-635.

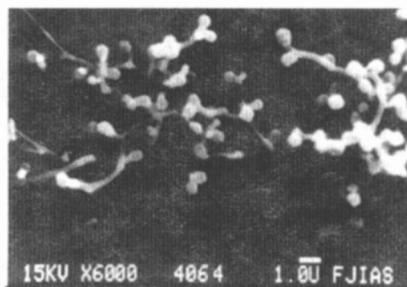


Fig. 1 Scanning electron micrograph of strain FIM 02-635

2.2 Physico-chemical properties of FW635I and FW635II

Two compounds were soluble in most organic solvents but not in petroleum ether, cyclohexane and water. The UV spectra of FW635I and FW635II showed absorption maxima at 248 and 262nm respectively. EFM-MS gave the molecular masses: 255, 137, 138 (M⁺), calculation 254 for FW635I; 271, 243, 153 (M⁺), calculation 270 for FW635II. The molecular formula of FW635I is C₁₅H₁₀O₄ and of FW635II is C₁₅H₁₀O₅, which were determined by using EFM-MS and NMR techniques (Table 1). The chemical shift in the ¹H and ¹³C NMR of FW635I and FW635II were shown in Table 2. On the basis of the above findings, compound FW635I and FW635II are identical with Daidzein and Genistein^[8] respectively. The chemical structure of FW635I and FW635II were shown in Fig. 2.

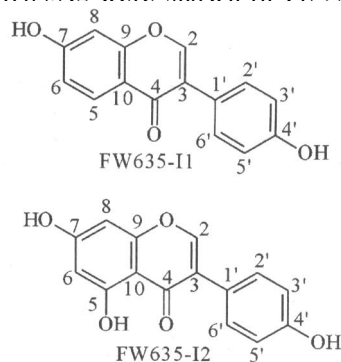


Fig. 2 Chemical structures of FW635I and FW635II

Table 1 Physico-chemical properties of FW635II and FW635I2

	FW635II	FW635I2
Appearance	Colourless, needle crystal	Colourless, needle crystal
Melting point	310-315	305-310
Solubility	Soluble	Soluble
	MeOH, EtOH, Me ₂ CO DMSO, Pyrimidine	MeOH, EtOH, Me ₂ CO DMSO, Pyrimidine
Insoluble	Petroleum ether, cyclohexane, H ₂ O	Petroleum ether, cyclohexane, H ₂ O
Molecular formula	C ₁₅ H ₁₀ O ₄	C ₁₅ H ₁₀ O ₅
Molecular weigh	254	270
EFMS (M ⁺)	255, 137, 118	271, 243, 153
UV max (MeOH, nm)	248	262
IR max (KBr, cm ⁻¹)	3234, 1631, 1596, 1517, 1461, 1384, 1307, 1272, 1240, 1194, 1172, 1097, 1046, 956, 889, 843, 790	3436, 1656, 1623, 1578, 1517, 1464, 1375, 1330, 1286, 1252, 1194, 1178, 1151, 1107, 1046, 882

Table 2 ¹H and ¹³C-NMR assignments () for FW635II and FW635I2

position	FW635II		FW635I2	
	C	H	C	H
2	153.0	8.18 (1H, s)	153.9	8.16 (1H, s)
3	118.5		122.8	
4	176.3		181.7	
5	128.8	8.47 (1H, d, J = 8.7 Hz)	159.8	
6	116.4	7.24 (1H, dd, J = 8.7, 2.7 Hz)	100.6	6.69 (1H, d, J = 2.4 Hz)
7	164.6		166.5	
8	103.6	7.13 (1H, d, J = 2.1 Hz)	95.1	6.78 (1H, d, J = 1.8 Hz)
9	159.9		159.2	
10	116.7		106.3	
1'	125.5		122.8	
2', 6'	131.6	7.83 (2H, d, J = 8.4 Hz)	131.6	7.75 (2H, d, J = 8.7 Hz)
3', 5'	116.7	7.30 (2H, d, J = 8.4 Hz)	116.8	7.31 (2H, d, J = 8.7 Hz)
4'	159.0		164.2	

2.3 Biological activities in vitro

2.3.1 Inhibitory effect on ConA induced splenocyte proliferation

The immunosuppressive activities of two compounds were examined by lymphocyte growth inhibitions. The splenic T-lymphocyte proliferation were significantly suppressed by 0.1 μmol · L⁻¹ of FW635II and FW635I2 respectively. Both compounds showed similar activity (IC₅₀ 1 μmol · L⁻¹, Fig. 3).

2.3.2 Inhibition of IL-2 Production

IL-2 production which was in the ConA induced splenocyte supernatant, was significantly inhibited by FW635II and FW635I2. Both compounds showed similar activity (IC₅₀ 3 μmol · L⁻¹, Fig. 4).

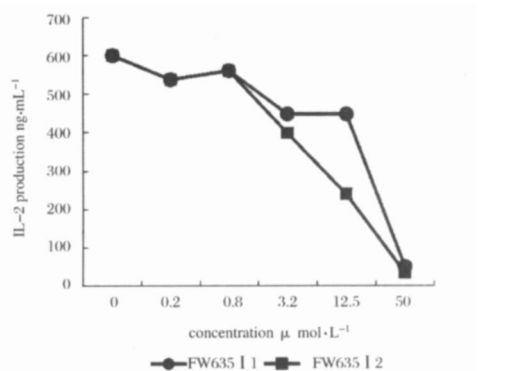


Fig. 3 Inhibitory effect on ConA induced splenocyte proliferation

2.3.3 Antitumor activity in vitro

Both compounds inhibited the human leukemia K562 cell proliferation with IC₅₀ value of 140 and 110 μmol · L⁻¹ for

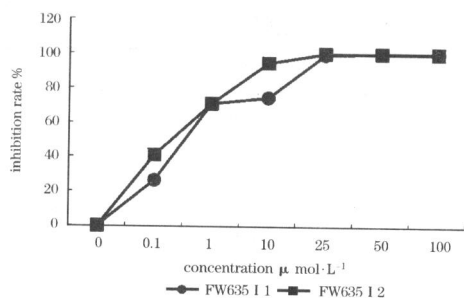


Fig. 4 Inhibition of L-2 production

FW635I1 and FW635I2 respectively .

2.3.4 Antimicrobial activities

Compounds, FW635I1 and FW635I2, showed no antimicrobial activities including bacteria and fungi.

3 Discussion

Isoflavones originated from plants, mainly from soy plant. The secondary metabolites extracted from the plants have several functions for great benefits to human health, for instances as estrogens, antiestrogens, cancer enzyme inhibitors and antioxidants. Although isoflavones were found in the soy plant, somewhat microorganisms including streptomyces, molds, pseudomonas were found to be able to produce these compounds^[9-11]. Two compounds FW635I1 and FW635I2 isolated from a marine *Micromonospora* sp. FIM 02-635 were identified with Daidzein and Genistein respectively and the producing strain *Micromonospora* sp. FIM 02-635 was classified as *Micromonospora carbonecea* FIM 02-635.

As far as we know only one species of the genus *Micromonospora*, *Micromonospora halophytica* isolated from terrestrial habitats produced Daidzein and Genistein^[10-14]. However we found a marine *Micromonospora carbonecea* FIM 02-635 is able to produce two important isoflavones, FW635I1 (Daidzein) and FW635I2 (Genistein), with immunosuppressive activities. Hopefully,

this would lead to the discovery the new immunosuppressants or other natural products by marine *Micromonospora* isolated from the relatively extreme environmental conditions.

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